SPLICE CHOICE ANTAGONISTS AS THERAPEUTIC AGENTS

Cross Reference To Related Patent Applications

This application is related to and claims priority on United States provisional patent application serial number 60/202,657 filed May 8, 2000.

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Field of the Invention

The invention relates to methods and reagents for influencing alternative RNA splicing in living cells. More particularly, the invention relates to novel means for influencing RNA splice choice in living cells using polynucleotide-based reagents that compete for binding sites in nucleotide binding proteins, and novel methods for using these reagents as therapeutics.

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Background of the Invention

The spatial and temporal coordination of gene expression during embryogenesis involves a variety of regulatory mechanisms, of which those acting at the transcriptional level have been most intensively studied (Davidson et al., 1998; Gellon and McGinnis,

1998; Gray and Levine, 1996; Mannervik et al., 1999). Less is known about mechanisms that control differential production and accumulation of specific proteins at various sites in the developing embryo at the post-transcriptional level, causing the RNA transcript to be spliced appropriately, or regulating transport of the spliced mRNA to the cytoplasm. Recent interest in the role of differential splicing in development and the factors and mechanisms by which it is accomplished (Chabot, 1996; Lopez, 1998), and a growing understanding of the determinants of nucleo-cytoplasmic transport of particular mRNAs (Piñol-Roma and Dreyfuss, 1992; Siomi and Dreyfuss, 1997; Weis, 1998), has set the stage for a systematic analysis of how these RNA processing factors contribute to regional and cell type specificity in the embryo.

The ribonucleoprotein hnRNP A1 is of particular interest in this regard, as it functions in both RNA splice site selection and nucleus-to-cytoplasm transport of mRNA. In its capacity as a splicing factor, this protein modulates 5' splice site selection in a group of gene products, some of which contain a well-characterized RNA sequence determinant (Burd and Dreyfuss, 1994). Among these are the pre-mRNAs of the HIV type 1 tat protein (Del Gatto-Konczak et al., 1999), FGF receptor 2 (FGFR2) (Del Gatto-Konczak et al., 1999), and hnRNP A1 itself (Chabot et al., 1997). In its role in nucleus-to-cytoplasm transport, hnRNP A1 acts as a "shuttle" protein (Piñol-Roma and Dreyfuss, 1992), and is characterized by a novel amino acid motif termed M9, which contains both nuclear localization and nuclear export activities (Michael et al., 1995; Siomi and Dreyfuss, 1995).

The known functions of hnRNP A1 as an RNA shuttle protein and in splice choice

selection are exerted in a gene product-specific fashion (Dreyfuss et al., 1993). The tissue-restricted spatiotemporal patterns in the protein's expression reported are therefore likely to be a causal component of the process by which cell types become distinctive from one another during organogenesis. A subclass of primary transcripts (including hnRNP A1 itself, Chabot et al., 1997; Mayeda et al., 1994) is differentially spliced by a process that depends on hnRNP A1. Control of splice choice appears to involve the antagonism of constitutive splicing factors such as SF2/ASF by members of the hnRNP A/B family of proteins (Mayeda and Krainer, 1992, Mayeda et al., 1993, 1994; Del Gatto-Konczak et al., 1999). Once spliced, these RNAs are transported into the cytoplasm by a process that involves hnRNP A1 and transportin 1 (Nakielny and Dreyfuss, 1998, Nakielny et al., 1999). This implies that the regulation of hnRNP A1 levels within living cells during development plays a key role in cell type diversification.

Earlier studies have surveyed the distribution of hnRNP A1 in a limited set of adult cell types (Kamma et al., 1995; Faura et al., 1995) including the developing germ cells of postnatal mice. A study by the inventors and their colleagues documenting the characterization of the sequence of chicken hnRNP A1 and its spatiotemporal and organ-specific expression during embryogenesis is hereby incorporated by reference in its entirety. (Bronstein et al., 2001).

hnRNP A1 protein is abundantly expressed in early stage epithelia such as skin, extraembryonic membranes, and neuroectoderm; epithelioid tissues, such as liver; as well as "secondary" epithelia and epithelioid tissues derived from mesenchymes, e.g., heart

muscle, skeletal muscle, kidney tubules, sinusoidal vascular endothelium, and precartilage condensations. It is not clear, however, whether this pattern represents an authentic expression theme, or simply the prevalence both of epithelioid tissues in the early embryo and of hnRNP A1 expression. (Bronstein et al., 2001).

The expression of hnRNP A1 in differentiating neuroectoderm and dorsal root ganglia broadly coincides with patterns of expression of members of the Hu class of RNA binding protein genes in the chicken (Wakamatsu and Weston, 1997) and it is significant in this regard that the Hu family of proteins results from extensive alternative splicing of Hu gene products during neurogenesis (Okano and Darnell, 1997). However, while the expression of the two RNA binding proteins may be partly overlapping, they are not entirely so: the typical DRG cell nuclei expressing hnRNP A1 are larger than those expressing Hu and vertebral body cartilage expresses hnRNP A1 but not Hu.

The transcription of natural antisense RNA cognate to exonic sequences of the hnRNP A1 gene in many of the same tissues that are producing sense transcript is an unusual phenomenon, but one that is not as rare as previously thought (Dolnick, 1997; Vanhee-Brossollet and Vaquero, 1998). Antisense RNA probably functions as a post-transcriptional inhibitor of gene expression (Knee and Murphy, 1997). This regulatory mechanism may be particularly relevant during development—natural antisense transcripts of several developmentally active growth factors—fibroblast growth factor-2 (Savage and Fallon, 1995), bone morphogenetic protein-2 (Feng et al., 1997), and transforming growth factor-2 (Coker et al., 1998)—have been detected at significant levels, the first two in

embryonic tissues. There is one previous report of differential expression of natural antisense RNA expression of a splicing factor gene (Sureau et al., 1997). Moreover, since hnRNP A1 can promote RNA-RNA strand annealing (Cobianchi et al., 1993; Idriss et al., 1994) it is itself a potential component of natural antisense regulatory mechanisms (Oberosler and Nellen, 1997).

The organ- and tissue-specific sense and antisense hnRNP A1 RNA expression patterns seen at different stages are consistent with the idea that antisense expression may be playing a regulatory role during development. For example, in kidney and liver virtually all cells at the early stages express the sense transcript. But whereas the protein product is also broadly distributed in liver the more limited distribution of the protein in kidney may be related to the more localized distribution of antisense RNA during development of this organ.

Because hnRNP A1 helps regulate nuclear-cytoplasmic transport and alternative splicing for well-defined classes of transcripts, its own regulation can provide the basis for post-transcriptional control of the partitioning of organ primordia into distinct gene expression domains. It is therefore significant that the hnRNP A1 gene is widely transcribed throughout the early embryo and its encoded protein is subject to numerous demonstrated and potential *autoregulatory* effects at the post-transcriptional level: it helps splice its own pre-mRNA, it may transport its own mRNA from the nucleus to the cytoplasm, and it may participate in the regulation of its own synthesis by its gene's antisense transcript. Small changes in the balance of any of these processes, or of other

possible but speculative ones, such as unmasking of maternally inherited hnRNP A1, or even transfer of the protein from one cell to another, could thus activate a post-transcriptional cascade leading to the local expression of hnRNP A1, and with it the expression of its target gene products.

The recent recognition that a large proportion of the genes constituting the human genome are alternatively spliced (Ewing and Green, 2000) (a recent estimate indicates that 38% of human mRNAs contain possible alternative splice forms; Bretta et al., 2000) highlights the centrality of the developmental regulation of hnRNP A1 and other nonconstitutive splicing factors in the generation of complexity in vertebrate organisms.

Classes of hnRNP proteins

In eukaryotes, heterogeneous nuclear RNAs (hnRNAs), which are the products of RNA polymerase II, are extensively processed to produce messenger RNAs (mRNAs). mRNA processing includes capping, splicing, and polyadenylation (Dreyfuss *et al.*, 1993) and involves the association of the hnRNAs with nuclear proteins collectively known as ribonucleoprotein (RNP) complexes (Dreyfuss *et al.*, 1993; Michael *et al.*, 1995). RNPs that directly bind to hnRNAs are classified as the hnRNPs and are involved in the splicing and shuttling of pre-mRNAs. Others are categorized into special classes such as small nuclear ribonuclear proteins (snRNPs) and include the U snRNPs (Bandziulis *et al.*, 1989; Dreyfuss *et al.*, 1993; Dreyfuss *et al.*, 1988; Luhrmann, 1990; Steitz, 1988; Zieve and Sauterer, 1990). The mature transcript produced from the hnRNA-hnRNP-snRNP complex is transported to the cytoplasm by specific hnRNPs where it may associate with yet another

set of RNPs involved in translational regulation and mRNA stability (Bandziulis *et al.*, 1989; Dreyfuss *et al.*, 1993; Luhrmann, 1990; Steitz, 1988; Zieve and Sauterer, 1990).

hnRNP proteins are highly conserved throughout the vertebrates, as well as having sequence homologies in the invertebrate *Drosophila* (Amrein *et al.*, 1988; Robinow and White, 1988) (Bell *et al.*, 1991; Dreyfuss *et al.*, 1993; Inoue *et al.*, 1990; Kay *et al.*, 1990; Roth *et al.*, 1991; Voelker *et al.*, 1990; Von Besser, 1990), and are the most abundant proteins found in the nucleus (Dreyfuss, 1986, Dreyfuss, 1993). In HeLa cells two-dimensional gel electrophoresis has resolved 20 major groups of proteins. These proteins are designated as the heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 (~34 kDa) to hnRNP U (~120 kDa), and categorized by structural motifs (Cobianchi, 1990; Dreyfuss *et al.*, 1993; Matunis *et al.*, 1992; Pinol-Roma *et al.*, 1988). Furthermore, sequence analysis has determined that hnRNPs have one or more RNA-binding modules referred to as the RNP motif or RNA Recognition Motif (RRM) in addition to at least one other auxiliary domain (Dreyfuss *et al.*, 1993). The RNP motif contains two consensus sequences, RNP1 and RNP2, within a domain of approximately 90 amino acid residues that are located about 30 amino acids from each other (Dreyfuss *et al.*, 1993; Dreyfuss *et al.*, 1988). The RNP 1 module is an octapeptide, Lys/Arg-Gly-Phe/Tyr-Gly/Ala-Phe-Val-X-Phe/Tyr (Adam *et al.*, 1986; Dreyfuss *et al.*, 1993), while the RNP2 module is a hexapeptide rich in aromatic and aliphatic amino acids and is less well conserved (Dreyfuss *et al.*, 1993; Dreyfuss *et al.*, 1988). Both of these consensus sequences are directly related to RNA binding (Dreyfuss *et al.*, 1993; Merrill *et al.*, 1988).

Functional and structural categories of human hnRNPs include:

(i) hn RNP A2/B1 complexes with the snRNPs and plays a role in splicing pre-mRNAs. Though localized in the nucleus of most tissues, A2 is also found in the cytoplasm of the squamous epithelium of the skin and the esophagus, and abundant amounts of A2 are found in the medulla, but not the cortex of the adrenal gland. Both A2 and B1 are found throughout spermatogenesis while A1 expression is repressed in spermatocytes (Kamma *et al.*, 1999).

(ii) hnRNP C1 is involved in the post-translation base change of cytosine to uracil in the apolipoprotein (apo) B mRNA which codes for the catalytic subunit APOBEC-1, a protein involved in splicesome assembly. C1 may regulate apoB mRNA editing thus restricting the activity of the catalytic subunit (Greeve *et al.*, 1998).

(iii) hnRNP D is involved in the immunoglobulin heavy chain recombination process by binding to the switching regions in conjunction with a B cell-specific duplex DNA binding factor (Dempsey *et al.*, 1999), while transcriptional regulation of the complement receptor 2 (CR2) is achieved by hnRNP D0B through its binding of both single and double stranded DNA (Tolnay *et al.*, 1997; Tolnay *et al.*, 1999).

(iv) hnRNP K may play a role in cytosine-rich pre-mRNA metabolism and cell cycle progression. Highly upregulated levels of K have been found in transformed keratinocytes (Dejgaard *et al.*, 1994).

(v) hnRNP H, H' are posttranslationally cleaved to produce the C-terminal proteins H(C) and H(C') both having a molecular weight of 35 kDa with localization

primarily in the nucleus. In contrast, hnRNP F varies with its localization depending on the cell type and is predominantly cytoplasmic in some cells which may be important its function (Honore *et al.*, 1999).

(vi) Autoantibodies of hnRNP A1, A2, B have been found in individuals with connective tissue diseases. In addition to the A/B proteins, hnRNP I has been found in patients with systemic sclerosis (SSc) and in particular, in individuals with pre–SSc or limited SSc. The A/B and I protein complexes may elicit autoimmune responses (Montecucco *et al.*, 1996).

(vii) The hnRNP L protein, having an unknown function, is found both as a component of the hnRNP complex as well as in discrete nonnucleolar structures of the nucleoplasm in HeLa cells (Pinol-Roma *et al.*, 1989).

(viii) Finally hnRNP R, an hnRNP P-like protein, was isolated from yet another individual with autoimmune symptoms and may be a component of subcellular particles that are found in autoimmune diseases (Hassfeld *et al.*, 1998). This protein may have some relationship to the gene product of the TLS/FUS gene, an RNA binding protein identical to hnRNP P2, and first identified as a fusion protein in human myxoid liposarcomas (Calvio *et al.*, 1995; Crozat *et al.*, 1993; Hassfeld *et al.*, 1998; Rabbitts *et al.*, 1993).

In addition, the hnRNP classes of RNA-binding proteins have been shown to be developmentally important in many embryonic tissues including the formation and maintenance of the nervous system (Dreyfuss *et al.*, 1993), sex determination in

Drosophila melanogaster (Bandziulis *et al.*, 1989; Del Gatto-Konczak *et al.*, 1999; Lynch and Maniatis, 1995; Lynch and Maniatis, 1996), neuronal splice activation (Del Gatto-Konczak *et al.*, 1999; Min, 1997) and maintenance (Dreyfuss, 1993), and epithelial/mesenchymal differentiation (Johnson and Williams, 1993). In *Drosophila*, the embryonic lethal abnormal visual (ELAV) system proteins are required for correct differentiation and maintenance of neurons. In mammals the ELAV-like neuronal RNA-binding proteins HuB, HuC, and HuD are implicated in neuronal development and differentiation in both the central and peripheral nervous systems (Akamatsu *et al.*, 1999; Kasashima *et al.*, 1999). In other systems such as the human immunodeficiency virus (HIV-1) hnRNPs are involved in regulating exon 2 of the *tat* splicing gene (Del Gatto-Konczak *et al.*, 1999; Si, 1997).

<u>hnRNP A1</u>

The hnRNP A1 protein contains two RNP consensus motifs, a glycine-rich auxiliary domain at its carboxy-terminus (Burd and Dreyfuss, 1994; Burd *et al.*, 1989; Buvoli *et al.*, 1990; Merrill *et al.*, 1988), as well as an RGG box, also at its carboxy-terminus (Kiledjian and Dreyfuss, 1992). In addition to these motifs, the hnRNP A1 class of proteins contain a nuclear localization signal, within a domain of approximately 38 amino acids at the carboxy-terminal region of the protein (Michael *et al.*, 1995). This motif, referred to as M9, is a novel nuclear localization signal (NLS)/nuclear export signal (NES) and is not homologous to the classical nuclear localization signal (NLS) found, for example in either the large T antigen of the SV40 virus or the bipartite basic NLS of nucleoplasmin

(Izaurralde *et al.*, 1997b; Kalderon *et al.*, 1984; Michael *et al.*, 1995; Robbins *et al.*, 1991; Weighardt *et al.*, 1995). The presence of the M9 motif allows hnRNPs to shuttle continuously between the nucleus and the cytoplasm (Dreyfuss *et al.*, 1993). hnRNPs of the A1, A2/B1, D, E, I and K classes have this capability, while those of the C1, C2, and U class are found restricted to the nucleus (Izaurralde *et al.*, 1997b; Michael *et al.*, 1995; Pinol-Roma and Dreyfuss, 1992). Furthermore, hnRNP A1 is found bound to the poly $(A)^+$ tail of RNA polymerase II transcripts in both the nucleus and the cytoplasm and data suggest that the hnRNP A1 protein is transported out of the nucleus with the mature message during the export process (Pinol-Roma and Dreyfuss, 1992). Fig. 1a shows the cDNA sequence designated SEQ ID NO:1 and Fig. 1b shows the amino acid sequence of chicken hnRNP A1 (indicated by CHKA1) designated SEQ ID NO:2 compared to the human hnRNP A1 amino acid sequence (indicated by HUMA1) designated SEQ ID NO:3.

Fig. 2 illlustrates the structure of the human core hnRNP proteins A1, $A1^B$, A2 and B1. The RNP-2 and RNP-1 conserved submotifs of RRM1 and RRM2, and the G domains of each protein are shown. hnRNP A1 and $A1^B$ or hnRNP A2 and B1 are identical except for extra amino acid regions indicated by boxes. The sequences of the RNP-1 and RNP-2 submotifs are aligned. The dots in the alignment indicate amino acid identities. All recombinant proteins are in authentic form except for post-translational modifications. The numbers indicate the position of amino acid residues from the initiation codon Met1. Based on published cDNA sequences (Burd, 1989; Buvoli, 1990). After Mayeda *et al.* (1994).

hnRNP A1 and splice choices

In a multi-step process, uracil rich small nuclear ribonuclear proteins (U snRNPs) in association with the core hnRNPs A1, A2, B1, B2, C1, C2, and C3 (classified by increasing molecular weight), bind to the pre-mRNAs in an ordered manner at specific sequences forming the spliceosome (Beyer *et al.*, 1977; Chung and Wooley, 1986; Del Gatto, 1996; Dreyfuss, 1986; Kumar *et al.*, 1986; Mayeda and Krainer, 1992). Alternative splicing allows for the functional and structural diversity of gene products by the addition or deletion of elements as small as a single amino acid (as seen in the Pax-3 and Pax-7 gene products) (Lopez, 1998). Additional means of obtaining protein variants from a single transcript in a cell-specific manner include splice activation and splice repression (Del Gatto-Konczak *et al.*, 1999).

Alternative splicing may involve the use of alternative 5' or 3' splice sites, optional exons, exclusive exons, or retained introns (Lopez, 1998). Except for intron retention, splicing patterns are under competitive control of splicing proteins (Lopez, 1998). Splice activation may involve multi-protein complexes on pre-mRNAs. An example of this is seen in the activation of the female specific *dsx* exon of *Drosophila melanogaster* by the female specific proteins, tra (transformer), tra-2 and SR (splice regulator proteins rich in arginine/glycine repeats) (Del Gatto-Konczak *et al.*, 1999; Lynch and Maniatis, 1995; Lynch and Maniatis, 1996; Wang *et al.*, 1998). In the mouse, the *c-scr* exon N1 is activated by the KSRP splicing factor (KH–type splicing regulator) (Min, 1997; Wang and Manley, 1997) which induces the assembly of five other proteins including hnRNP F (a pre-mRNA splicing factor which is associated with the TATA-binding protein, essential for transcription

initiation (Del Gatto-Konczak *et al.*, 1999; Min, 1997; Yoshida *et al.*, 1999). This multiprotein complex activates the intronic splicing enhancer that splices the neuronal specific c-*scr* N1 exon *in vitro* (Del Gatto-Konczak *et al.*, 1999; Min, 1997).

Splice repression involves protein binding to an intronic 3' splice site and is seen in the female-specific Sxl protein of *Drosophila*. This interaction effectively blocks U2 snRNP and U2AF (U2 snRNP auxiliary factor) (Del Gatto, 1996; Del Gatto-Konczak *et al.*, 1999; Lopez, 1998; Valcarcel *et al.*, 1993). Other protein complexes may use exon sequences for splice repression.

Vertebrate genes including the human *fibroblast growth factor receptor 2* gene (*fgfr2*), and the human immunodeficiency virus type 1 (HIV-1) *tat* gene contain exons that have sequences acting as exonic splice silencers (ESS) (Amendt *et al.*, 1994; Amendt *et al.*, 1995; Baba-Aissa *et al.*, 1998; Caputi *et al.*, 1994; Caputi *et al.*, 1999; Del Gatto, 1995; Del Gatto, 1996; Del Gatto-Konczak *et al.*, 1999; Gallego *et al.*, 1996; Graham *et al.*, 1992; Si, 1997).

The ESS of the human *FGFR2* pre-mRNA contains a UAGG sequence in the *kgfr* exon (keratinocyte growth factor receptor-exon 8) (Del Gatto, 1996; Del Gatto and Breathnach, 1995; Del Gatto-Konczak *et al.*, 1999). This sequence has homology to the high affinity consensus sequence 5'-UAGGGA/U-3' recognized by hnRNP A1 (Del Gatto-Konczak *et al.*, 1999). In *in vitro* studies, Del Gatto-Konczak *et. al.* (1999) have demonstrated that hnRNP A1 can modulate splice choices by binding to a 10 mer ESS designated S10 (5'-UAGGGCAGGC-3') or to a 6 mer ESS designated S6 (5'-UAGGGC-3').

In *in vitro* studies, RNA molecules containing the splicing silencer sequence from the human fibroblast receptor 2 *kgfr* exon *(IIIb)* were capable of directing splice choice selection by the recruitment hnRNP A1 (Del Gatto-Konczak *et al.*, 1999). When the following point mutations were introduced into the S6 ESS UCGGGC or UACGGC a two-fold decrease in hnRNP A1 binding was detected (Del Gatto-Konczak *et al.*, 1999). Furthermore, it was determined that the targeting of hnRNP A1 to the ESS domain was through the glycine-rich motif at the C-terminus of the protein. In the human hnRNP A1 protein, the glycine-rich domains are found between residues 189-320: the RGG motif is specifically located at residues 189-247, followed by another glycine-rich motif from residues 239-320 (Del Gatto-Konczak *et al.*, 1999). Silencing of the *k-sam* (*kgfr*) exon in these *in vitro* studies required the entire glycine-rich motif. By examining the corresponding sequence in the chicken *kgfr* exon (*IIIb* exon 8) of *fgfr2* it has been determined that the sequence corresponding to the human ESS is 5'-UAGGGAGGGC-3'.

Studies involving hnRNP A1 proteins demonstrated that it is capable of promoting RNA molecules to base pair into double stranded structures, therefore influencing pre-mRNA splicing by snRNPs (Burd and Dreyfuss, 1994; Buvoli *et al.*, 1992; Eperon *et al.*, 1993; Kumar and Wilson, 1990; Munroe and Dong, 1992; Pontius and Berg, 1990; Portman and Dreyfuss, 1994). In *in vitro* assays hnRNP A1, as well as the RNA binding protein splicing factor 2 (ASF/SF2) (a member of the SR nuclear phosphoprotein family) were capable of making splice choices at the 5' splice site of pre-mRNAs that contain multiple 5' splice sites and are essential for constitutive splicing (Caceres *et al.*, 1997;

Caceres *et al.*, 1998; Del Gatto, 1996; Fu, 1995; Ge and Manley, 1990; Krainer *et al.*, 1990; Manley and Tacke, 1996; Mayeda *et al.*, 1993; Mayeda and Krainer, 1992; Mayeda *et al.*, 1994; Munroe and Dong, 1992; Zahler *et al.*, 1993).

In vitro studies suggest that hnRNP A1 and ASF/SF2 may act antagonistically and that the hnRNP A/B family of splicing proteins regulates the SR family both *in vitro* and *in vivo* (Caceres *et al.*, 1998; Caceres *et al.*, 1994; Mayeda and Krainer, 1992; Yang *et al.*, 1994). In *in vitro* experiments, excess hnRNP A1 favored the distal 5' splice site, in contrast to excess ASF/SF2 favoring proximal 5' splice sites in a concentration-dependent manner resulting in alternate splicing patterns of many genes in specific cell types (Del Gatto, 1996; Mayeda *et al.*, 1993; Mayeda and Krainer, 1992; Mayeda *et al.*, 1994; Munroe and Dong, 1992). Burd and Dreyfuss (1994) have shown that the consensus sequence 5'-UAGGGA/U-3' is a high affinity binding site of hnRNP A1 and that this sequence is similar to the 5' and 3' splice sites in vertebrate pre-mRNAs. In addition, the ability of hnRNP A1 to bind to this consensus sequence increased if it was duplicated and separated by two nucleotides, resulting in a dissociation constant of 1×10^{-9} M. While hnRNP A1 proteins are capable of binding to other pre-mRNA sites, binding affinity varies greatly over a >100 fold range, therefore classifying these proteins as sequence specific RNA binding proteins (Burd and Dreyfuss, 1994).

hnRNP A1 is also involved in self-splicing. The 4.6 kb human hnRNP A1 mRNA containing 10 exons encodes for the 34 kDa hnRNP A1 protein. The pre-mRNA for hnRNP A1 can be differentially spliced to produce the A1 form and $A1^B$ form (Buvoli *et al.*, 1990).

It has been shown that the human hnRNP $A1^B$ protein (Fig. 2) with an apparent molecular weight of 38 kDa, corresponds to the protein previous designated as hnRNP B2 (Buvoli *et al.*, 1990). The $A1^B$ splice variant which contains an extra exon in the C-terminal region glycine-rich region (156bp; 52 amino acids) has a higher affinity for ssDNA than the 34kDa form though its abundance in the cell is only ~5% that of hnRNP A1 (Buvoli *et al.*, 1990).

More recently, Blanchette and Chabot (1997) have shown that alternative splicing of the hnRNP A1 pre-mRNA yields the A1 and $A1^B$ forms via 5' splice selection and exon skipping, and that this process requires conserved elements. Studies have shown that the addition of the alternate exon 7B in the mature mRNA produces the hnRNP $A1^B$ protein (Buvoli *et al.*, 1990). Furthermore, Blanchette and Chabot have demonstrated that the conserved intron element (CE1) upstream from exon 7B favors distal 5' splice site selection. SR proteins, including SF2, which favor the proximal 5' splice selection site, require U1 snRNP and U2AF when involved in the 5' splice site stimulation of a 3' splice site, as seen in the male specific 3' splice site of *tra* in *Drosophila* (Blanchette and Chabot, 1997; Valcarcel *et al.*, 1993). Interestingly, the CE1 element does not interfere with U1 snRNP binding and led to the discovery of an additional element CE610, which is located downstream from exon 7B. CE610 is also involved in distal 5' splice site selection by secondary structure formation and exon skipping (Blanchette and Chabot, 1997). Since the SR family of splice selection proteins and hnRNP A1 act antagonistically for 5' splice choices, where the SRs choose the 5' proximal site and the hnRNPs the 5' distal site (Weighardt *et al.*, 1996), hnRNP A1 may be involved in modulating its own splicing

(Blanchette and Chabot, 1997; Chabot *et al.*, 1997; Del Gatto-Konczak *et al.*, 1999; Mayeda *et al.*, 1994).

Fibroblast growth factors (FGFs), fibroblast growth factor receptors (FGFRs), and FGFR-2 splice variants

Fibroblast growth factors (FGFs) are important mitogens in both cell proliferation and differentiation, but in some cases may act as antagonists and inhibit differentiation. Examples of FGF induced differentiation are seen in the stimulation of pre-adipocyte fibroblasts (Broad and Ham, 1983; Johnson and Williams, 1993; Serrero and Khoo, 1982), and hippocampal neurite outgrowth (Johnson and Williams, 1993; Walicke *et al.*, 1986). Developmental roles have been demonstrated in embryonic mesodermal induction in *Xenopus* (Kimelman and Kirschner, 1987; Slack *et al.*, 1987), and the inhibition of differentiation of myotubes has been shown in skeletal muscle (Linkhart *et al.*, 1981). In addition to acidic FGF (aFGF or FGF1) and basic FGF (bFGF or FGF2), the family of FGFs, including keratinocyte growth factor (KGF) have been shown to stimulate the proliferation of mesenchymal and neuroectodermal cell types (Burgess and Maciag, 1989; Johnson and Williams, 1993). Using immunohistochemical analysis on chick embryo sections, FGF2 has been localized to the heart, myotome, limbs and muscles (Han, 1997; Joseph-Silverstein, 1989) as well as to the notochord, neural tissue, gut cells, and tubules in the mesonephric and metanephric kidneys (Dono and Zeller, 1994; Han, 1997). In addition to the previously mentioned tissues, Han (1997) localized this mitogen to the developing pharyngeal arches, specifically the maxilla and mandible. FGF2 plays an

important role in morphogenesis and pattern formation in the vertebrate limb (Han, 1997; Noji *et al.*, 1993; Riley *et al.*, 1993; Savage *et al.*, 1993), as well as in kidney development (Dono and Zeller, 1994; Han, 1997).

Receptors for the 19 known fibroblast growth factors (FGFs) (Hu *et al.*, 1998; Ohbayashi *et al.*, 1998) include the tyrosine kinase fibroblast growth factor receptors (FGFRs) (Johnson and Williams, 1993), the CFR receptor or cytosine rich FGFR, (Burrus and Olwin, 1989) and the heparan sulfate proteoglycans (HSPGs). In chicken, the genes for *fgfrs1, 2,* and *3* and 4 (*fgfr-related kinase* or *frek*) as well as the *kgfr* (*exon IIIb-keratinocyte growth factor receptor*) and *bek* (*exon IIIc-bacterial expressed kinase*) splice variants for receptors 1 and 2 have been cloned (Szebenyi *et al.*, 1995). The vertebrate FGFRs contain the domains as described by Johnson and Williams, (1993). Modifications of FGFR isoforms are due to alternative splicing of the pre-mRNAs for each gene. In a schematic representation of human FGFR1, the extracellular region of the molecule has the following domains including a signal peptide region at its N-terminus, followed by three immunoglubulin-like (Ig-like) domains with an acid box between domain I and II. A membrane-proximal region precedes the transmembrane (TM) region. On the intracellular side, two tyrosine kinase domains that are separated by a kinase insert follow a juxtamembrane (JM) domain, and at the C-terminus is a C-tail domain. The third Ig-loop of FGF receptor 2 is involved in the chondrogenic process and can contain either the *IIIa* and *IIIb* (*kgfr*) or *IIIa* and *IIIc* (*bek*) exonic sequences (Johnson and Williams, 1993).

Using chick limb micromass culture, Szebenyi *et al.* (1995) have looked at changes in the expression of the FGFRs in differentiated cartilage and have found transcripts for *fgfr1* in undifferentiated proliferating mesenchyme, *fgfr2* in precartilage condensations, and *fgfr3* in differentiating cartilage nodules suggesting spatiotemporal regulation in limb development. Binding of the FGFs to their receptors plays an important role in limb development through the regulation of cell survival, proliferation, and precartilage cell differentiation (Fallon *et al.*, 1994; MacCabe *et al.*, 1991; Niswander *et al.*, 1993; Schofield and Wolpert, 1990; Szebenyi *et al.*, 1995; Watanabe and Ide, 1993).

The messenger RNA splice variants *IIIb* (*kgfr*) and *IIIc* (*bek*) from *fgfr1* and *fgfr2*, as well as *fgfr3* were detected in nuclease protection assays on chicken limbs (Szebenyi *et al.*, 1995). In addition, micromass cultures of stage 23-24 wing buds and *in situ* hybridization of stage 18, 23, 26, and 36 wings showed a spatial distribution of messenger RNA for *fgfr1*, *2*, and *3*. Furthermore, the probes for *fgfr1* and *2* contained sequences for both the *kgfr* and *bek* splice variants and did not allow for the *in vivo* or *in vitro* detection of either of these isoforms. This is critical since there is a cell type specific role for the fibroblast growth factor receptor 2 isoforms, (FGFR2) kgfr (exon 8-*IIIb*) and bek (exon 9-*IIIc*), in precartilage differentiation. In addition, fibroblast growth factors (FGFs) influence cell function in a tissue-specific or developmental manner that can lead to defects such as craniosynostosis and syndactyly (Del Gatto, 1996; Mayeda and Krainer, 1992; Oldridge *et al.*, 1999). This differential splicing of the pre-mRNAs produced by a single gene allows for the production of splice variants and results in forms that respond to the different growth

factor isoforms in a highly specific manner. Cells that will differentiate into epithelia splice only the *kgfr* exon, while mesenchymal cells, including fibroblasts, as well as other cell types including endothelial cells, splice the 5' distal *bek* exon (Del Gatto, 1996; Fallon *et al.*, 1994; Johnson and Williams, 1993; Rubin *et al.*, 1989; Szebenyi *et al.*, 1995).

Mutations in the various receptors cause various skeletal defects (Oldridge *et al.*, 1999). Pfeiffer syndrome, a mutation in the *fgfr1* gene, presents with craniosynostosis as well as limb defects; Crouzon syndrome, a result of a mutation in the *fgfr2* gene, presents with limb abnormalities; and type II achondroplasia or dwarfism, is caused by a mutation in the *fgfr3* gene (Szebenyi *et al.*, 1995). Apert syndrome or acrocephalosyndactyly type 1, presents with head, hand, and foot abnormalities (Anderson *et al.*, 1999). Oldridge *et al.* (1999) looked at mutations in the *fgfr2* gene of 260 unrelated Apert syndrome patients and found that 258 individuals have a missense mutation in exon 7 which lies between the 2nd and 3rd Ig-like loop domains. The remaining two individuals had an ~ 360 bp insertion of an *Alu*-element either 5' to exon 9 or within exon 9, which arose as *de novo* mutations in the paternal chromosome (Oldridge *et al.*, 1999). Exon 9 corresponds to the 3rd Ig-like loop domain and contains the *bek* (*IIIc*) sequence (Oldridge *et al.*, 1999). In early studies involving the role of the *fgfr2* splice variants *IIIb* (exon 8) and *IIIc* (exon 9), Rubin *et al.* (1989) found that keratinocytes expressed the FGFR2 IIIb isoform and were stimulated by KGF. In addition, fibroblasts and endothelial cells expressed the FGFR2 IIIc isoform, and responded to FGF2 (bFGF) (Johnson and Williams, 1993; Rubin *et al.*, 1989). In an RNA analysis of fibroblasts obtained from two Apert and two Pfeiffer syndrome patients having

mutations in exon 9, severity of limb abnormalities directly corresponded to ectopic expression of the of the *IIIc-kgfr* form of the FGFR2. These data provided evidence of the role of signal transduction pathways through the KGFR form of the receptor in relation to syndactyly in Apert syndrome (Oldridge *et al.*, 1999; Park *et al.*, 1995; Wilkie *et al.*, 1995).

Fig. 4 illustrates FGFR2 with positional mutations, polymorphic nucleotides, and primers using in the Oldridge study. Top shows leader sequence (L), acid box (A), three Ig-like domains (*Igl, IgIII,* and *IgIII*), a transmembrane region (TM), and a split tyrosine-kinase domain (TK1 and TK2). Exons 8 and 9 encode for the alternative splice variants of the second a half of the *IgIII* domain, which is depicted by the *IgIIIb* (*kgfr* isoform)and *IgIIIc* (*bek* isoform) respectively. Positional mutations of two Apert syndrome patients (1 and 2) with *Alu* insertions, as well as two patients with Pfeiffer syndrome (3 and 4) with nucleotide substitutions are also shown. After Oldridge *et al.*, (1999)

Alternative splicing

An embodiment of the present invention describes a method and reagents that influence alternative splicing in living cells. Alternative splicing is a mechanism by which a single gene may eventually give rise to several different proteins. Alternative splicing is accomplished by the concerted action of a variety of different proteins, termed "alternative splicing regulatory proteins," that associate with the pre-mRNA in the cell nucleus, and cause distinct alternative exons to be included in the mature mRNA. These alternative forms of the gene's transcript give rise to distinct isoforms of the specified protein. The virulence of the HIV virus associated with AIDS depends on particular alternative splice

choices, and several cancers, rheumatoid and osteoarthritis, and other inflammatory diseases, exhibit aberrant splice choices when compared to corresponding non-diseased tissues.

An embodiment of the present invention describes a novel means for influencing splice choice in living cells using polynucleotide-based reagents that compete for binding sites in alternative splicing regulatory proteins, and novel methods for using these reagents as therapeutics.

An embodiment of the present invention contains the following novel aspects, which will be taken up in order:

1. A novel method for influencing splice choice in living cells using polynucleotide-based reagents that compete for binding sites in alternative splicing regulatory proteins.

Sequences in pre-mRNA molecules that bind to alternative splicing regulatory proteins can be found in introns or exons, and are known by the terms intronic splicing silencers or enhancers, and exonic splicing silencers or enhancers (ISS, ISE, ESS, ESE). No published paper in the Medline database reports the introduction into living cells of polynucleotide-based competitors for ISS, ISE, ESS, or ESE binding sites in alternative splicing factors. Burd, C.G., and Dreyfuss, G. (1994) identified a 20-mer RNA sequence that binds the alternative splicing factor hnRNP A1, but this was work done with isolated protein and nucleic acid components, not within living cells. Blanchette, M., and Chabot, B. (1999) and Breathnach and co-workers (Del Gatto, F., and Breathnach, R. , 1995; Del

Gatto, F., Gesnel, M.C., and Breathnach, R., 1996; Del Gatto, F., Plet, Al, Gesnel, M.C., Fort, C., and Breathnach, R., 1997; Del Gatto-Konczak, F., Olive, M., Gesne, M.C., and Breathnach, R., 1999; have investigated the effects of various ISS, ISE, ESS, ESE-related sequences in splice choice, but all these experiments have been done in cell-free extracts, not within living cells.

2. Novel methods for using the reagents described above as therapeutics.

Although it has been recognized for some time that the life cycle of the AIDS virus HIV involves alternative splicing (Amendt, B.A., Si, Z.H., and Stoltzfus, C.M., 1995; Si, Z., Amendt, B.A., and Stoltzfus, C.M., 1997; Si, Z. H., Rauch, D., and Stoltzfus, C.M., 1998; Del Gatto-Konczak, F., Olive, M., Gesnel, M.C., and Breathnach, R., 1999; none of these, nor any other, studies propose treating the disease with competitors of ISS, ISE, ESS, or ESEs.

However, it is likely that if agents that competed with alternative splicing regulatory proteins such as hnRNP A1 for the HIV tat protein ESS were introduced into HIV infected cells, as shown in the Dissertation of one of the inventors "Avian hnRNP A1, an mRNA Shuttle Protein-Exon Splicing Silencer: Developmental Regulation and Role in Chondrogenesis" (Department of Cell Biology and Anatomy, New York Nedical College), which is herein incorporated by reference in its entirety, indicates that the method is feasible and effective, and that the the viral infection would be attenuated (Purcell, D.F., and Martin, M.A., 1993). Indeed, splicing of HIV-1 pre-mRNA must be inefficient to provide a pool of unspliced messages which encode viral proteins and serve as genomes for new

virions (Caputi, M., Mayeda, A., Krainer, A.R., and Zahler, A.M., 1999), and virus production is arrested in a natural HIV variant that has an aberrant ESS (Wentz, M.P., Moore, B.E., Cloyd, M.W., Berget, S.M., and Donehower, L.A., 1997).

With regard to cancer, it has been found that certain tumors, such as mammary carcinomas (Stickeler, E., Kittrell, F., Medina, D., and Berget, S.M., 1999) and colon adenocarcinomas (Ghigna, C., Moroni, M., Porta, C., Riva, S., and Biamonti, G., 1998) contain levels of hnRNP A1 and other alternative splicing regulatory proteins that are altered relative to related normal tissues. Moreover, this abnormality is reflected in aberrant splicing patterns of certain alternatively spliced gene products, such as the cell adhesive protein CD44, although the specific role of splice variants of CD44 in tumorigenicity and metastasis is unresolved (Sneath, R.J., and Mangham, D.C., 1998).

The neoplastic state is characterized by numerous other gene products that show aberrant alternative splicing patterns. These include the extracellular matrix protein fibronectin (Midulla, M. Verma, R., Pitnatelli, M., Ritter, M.A., Courtenay-Luck, N.S., and George, A.J., 2000), the proteolytic enzyme cathepsin B (Keppler, D., and Sloane, B.F., 1996), the breast cancer susceptibility gene BRCA2 (Bieche, I., and Lidereau, R., 1999), and the apoptosis-associated gene products Bcl-x (Xerri, L., Hassoun, J., Devilard, E., Birnbaum, D., and Birg, F., 1998), Bax (Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J., 1993), and caspase 2 (Ich-1) (Jiang, Z.H., and Wu, J.Y., 1999). The apparent causal relationship of some of these aberrant splicing patterns to the neoplastic state, coupled with the emerging evidence that tumors express abnormal levels of alternative splicing

regulatory proteins, suggest that treatment with agents that specifically inhibit these regulatory proteins, such as those methods and reagents disclosed and claimed herein, represent a promising approach to cancer therapy.

Inflammatory diseases such as rheumatoid and osteoarthritis also involve protein (e.g., CD44) that exhibit abnormal alternative splicing patterns (Croft, D.R., Dall, P., Davies, D., Jackson, D.G., McIntyre, P., and Kamer, L.M., 1997; Boyle D.L., Shi, Y., Gat, S., and Firestein, G.S., 2000), and it is reasonable to hypothesize that the resulting aberrant proteins, among which are secreted and cell surface molecules, contribute to the immune-mediated manifestations of these diseases. Again, these data suggest that treatment with agents that specifically target alternative splicing factors represent a promising therapeutic approach.

Several publications have suggested using an antisense strategy to alter splicing patterns as therapeutics for cancer and ceratin other diseases (but not AIDS) (Sierakowska, H., Gorman, L., Kang, S.H., and Kole, R. (2000); Mercatante, D., and Kole, R., 2000). The invention described herein is not an antisense strategy, and has many advantages over such a strategy.

Current treatment of AIDS uses multiple reagents (AXT, protease inhibitors) directed against different biological functions of HIV. The method and reagents according to an embodiment of the present invention are directed against a distinct cell-virus interactive function, alternative splicing, and should add productively to the spectrum of

agents available for treatment of this disease. Current treatment for cancer involves the use of agents that are frequently highly toxic and nonspecific. The method and reagents according to an embodiment of the present invention will constitute therapeutics with high specificity for a biological function, alternative splicing, that is aberrant in many cancers.

Summary of the Invention

In response to the foregoing challenge, Applicants have developed an innovative, economical method of modifying the activity of nucleotide binding proteins within cells comprising introducing into cells polynucleotide sequences capable of binding to nucleotide binding proteins, binding within cells the polynucleotide sequences to the nucleotide binding proteins, and modifying within cells the activity of the nucleotide binding proteins with the binding.

The polynucleotide sequences may be introduced into the cells by electroporation, by applying the polynucleotide sequences to the surface of the cells, by packaging the polynucleotide sequences in liposomes, and by applying the polynucleotide sequences to the surface of the cells along with a detergent.

The cells may be human cells, tissue culture cells, non-human cells, non-human mammalian cells, avian cells, and non-human tissue culture cells.

The polynucleotide sequences may comprise RNA, isolated and purified RNA molecules, synthetic RNA molecules, and synthetic RNA analogs (chemical compositions similar to RNA), DNA, isolated and purified DNA molecules, synthetic DNA molecules, and

synthetic DNA analogs (chemical compositions similar to DNA). The polynucleotide sequences may be single-stranded or double-stranded.

The step of modifying within cells the activity of the nucleotide binding proteins may comprise regulating the activity of the nucleotide binding proteins, reducing the activity of the nucleotide binding proteins, and/or blocking the activity of the nucleotide binding proteins. The binding of the polynucleotide sequences may be reversible or irreversible.

The method may further comprise the step of causing an effect within cells in the processing of RNA by modifying the activity of the nucleotide binding proteins.

The method may further comprise the step of determining the effect in the processing of RNA by the resulting phenotypic characteristics of the cells, and/or by Northern blot analysis of cell extracts.

Another embodiment of the present invention is a method of modifying the activity of RNA binding proteins within cells comprising introducing into cells polynucleotide sequences capable of binding to RNA binding proteins, binding within cells the polynucleotide sequences to the RNA binding proteins, and modifying within cells the activity of the RNA binding proteins with said binding. The method may further comprise the step of causing an effect within cells in the processing of RNA by modifying the activity of the RNA binding proteins.

Another embodiment of the present invention is a method of modifying the activity of RNA alternative splicing regulatory proteins within cells comprising: a) introducing into cells polynucleotide sequences capable of binding to RNA alternative splicing regulatory

proteins; b) binding within cells the polynucleotide sequences to the RNA alternative splicing regulatory proteins; and c) modifying within cells the activity of the RNA alternative splicing regulatory proteins with said binding.

Another embodiment of the present invention is a method of modifying the activity of hnRNP proteins within cells comprising: a) introducing into cells polynucleotide sequences capable of binding to hnRNP proteins; b) binding within cells the polynucleotide sequences to the hnRNP proteins; and c) modifying within cells the activity of the hnRNP proteins with the binding.

Another embodiment of the present invention is method of modifying the activity of hnRNP A1 proteins within cells comprising: a) introducing into cells polynucleotide sequences capable of binding to hnRNP A1 proteins; b) binding within cells the polynucleotide sequences to the hnRNP A1 proteins; and c) modifying within cells the activity of the hnRNP A1 proteins with the binding.

Another embodiment of the present invention is a method of modifying the activity of nucleotide binding proteins within cells comprising: a) introducing into cells polynucleotide sequences complementary to binding sites of nucleotide binding proteins; b) binding within cells the polynucleotide sequences to the nucleotide binding proteins; and c) modifying within cells the activity of the nucleotide binding proteins with the binding. The nucleotide Binding proteins may be RNA binding proteins, RNA alternative splicing regulatory proteins, hnRNP proteins, and/or hnRNP A1 proteins.

An alternative embodiment of the present invention is a method of modifying the activity of nucleotide binding proteins within cells comprising: a) introducing into cells polynucleotide sequences that bind to nucleotide binding proteins; b) binding within cells the polynucleotide sequences to the nucleotide binding proteins; and c) modifying within cells the activity of the nucleotide binding proteins with the binding.

An alternative embodiment of the present invention is a method of influencing splice choice in RNA within cells comprising: a) introducing into cells polynucleotide sequences that bind to nucleotide binding proteins; b) binding within cells the polynucleotide sequences to the nucleotide binding proteins; and c) modifying within cells the activity of the nucleotide binding proteins with the binding.

An alternative embodiment of the present invention is a composition comprising a non-naturally occuring polynucleotide sequence that binds within cells to an hnRNP A1 protein of Seq. ID No. 2 and modifies the activity of the hnRNP A1 protein. The non-naturally occuring polynucleotide sequence may be a synthetic polynucleotide sequence, and/or a polynucleotide sequence analog. The non-naturally occuring polynucleotide sequence may bind to an hnRNP A1 protein of Seq. ID No. 2 under physiological conditions and modify the activity of the hnRNP A1 protein. The non-naturally occuring polynucleotide sequence may influence RNA splice choice within cells by modifying the activity of the nucleotide binding proteins.

An alternative embodiment of the present invention is a composition comprising a non-naturally occuring polynucleotide sequence bound to an hnRNP A1 protein.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention as claimed. The accompanying drawings, which are incorporated herein by reference, and which constitute a part of this specification, illustrate certain embodiments of the invention, and together with the detailed description serve to explain the principles of the present invention.

Brief Description of the Drawings

Fig. 1a is a listing of the full length cDNA sequence of chicken hnRNP A1 designated as SEQ ID NO:1. Uppercase letters indicate the open reading frame designated as SEQ ID NO:4.

Fig. 1b is a listing of the translation product of SEQ ID NO:4, designated as SEQ ID NO:2 aligned with the amino acid sequence of human hnRNP A1 designated as SEQ ID NO:3

Fig. 2 is a schematic representation of the structure of the human core hnRNP proteins A1, A1B, A2, and B1.

Figs. 3a, b, and c are photographs and x-rays illustrating syndactyly of hand and feet.

Fig. 4 is a schematic representation of the FGFR2 genetic map.

Fig. 5a is a photomicrograph of chicken leg bud mesenchymal cells transfected with FGFR2 exon 9 sense strand RNA and exon 8 sense strand RNA.

Fig. 5b is an x-ray of a developing chicken leg bud transfected with FGFR2 exon 8.

Fig. 5c is a Northern blot using poly A RNA from leg bud mesenchyme transfected with FGFR2 exon 8 or exon 9 sense strand RNA.

Detailed Description of the Preferred Embodiments

The present invention comprises a method of modifying the activity of nucleotide binding proteins within cells. A preferred embodiment of the method of the present invention comprises introducing into cells polynucleotide sequences that bind to nucleotide binding proteins, modifying within cells the activity of the nucleotide binding protein by binding the polynucleotides to the nucleotide binding proteins, causing an effect within the cells in the processing of RNA by modifying the activity of the nucleotide binding proteins, and determining the effect in the processing of RNA resulting from the modification of the activity of the nucleotide binding proteins. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide by electroporation. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequences to the surface of the cells packaged in liposomes. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells along with a detergent.

The step of modifying within cells the activity of the nucleotide binding proteins may further comprise regulating the activity of the nucleotide binding protein. The step of modifying within cells the activity of the nucleotide binding proteins may further comprise reducing the activity of the nucleotide binding proteins. The step of modifying within cells the activity of the nucleotide binding proteins may further comprise blocking the activity of the nucleotide binding proteins. The step of modifying within cells the activity of the nucleotide binding proteins may further comprise binding the polynucleotides either reversibly, or irreversibly.

As embodied herein, the step of determining the effect in the processing of RNA may further comprise determining the effect by phenotypic characteristics of the cells. The step of determining the effect in the processing of RNA may further comprise determining the effect by Northern blot analysis of cell extracts.

As embodied herein, the cells may further comprise tissue culture cells, and non-human tissue culture cells. The cells may also further comprise non-human cells, non-human mammalian cells, and avian cells.

As embodied herein, the polynucleotide sequences may further comprise isolated and purified RNA molecules, synthetic RNA molecules, and synthetic RNA analogs. The polynucleotide sequences may be single stranded.

An alternative preferred embodiment of the present invention is a method of modifying the activity of RNA binding proteins within cells comprising introducing into cells polynucleotide sequences that bind to RNA binding proteins, modifying within cells the

activity of the RNA binding proteins by binding the polynucleotides to the RNA binding proteins, causing an effect within cells in the processing of RNA by modifying the activity of the RNA binding proteins, and determining the effect resulting from the modification of the activity of the RNA binding proteins. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide by electroporation. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequences to the surface of the cells packaged in liposomes. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells along with a detergent.

The step of modifying within cells the activity of the RNA binding proteins may further comprise regulating the activity of the RNA binding proteins. The step of modifying within cells the activity of the RNA binding proteins may further comprise reducing the activity of the RNA binding proteins. The step of modifying within cells the activity of the RNA binding proteins may further comprise blocking the activity of the RNA binding proteins. The step of modifying within cells the activity of the RNA binding proteins may further comprise binding the polynucleotides either reversibly, or irreversibly.

As embodied herein, the step of determining the effect in the processing of RNA may further comprise determining the effect by phenotypic characteristics of the cells. The

step of determining the effect in the processing of RNA may further comprise determining the effect by Northern blot analysis of cell extracts.

As embodied herein, the cells may further comprise tissue culture cells, and non-human tissue culture cells. The cells may also further comprise non-human cells, non-human mammalian cells, and avian cells.

As embodied herein, the polynucleotide sequences may further comprise isolated and purified RNA molecules, synthetic RNA molecules, and synthetic RNA analogs. The polynucleotide sequences may be single stranded.

An alternative preferred embodiment of the present invention is a method of modifying the activity of RNA alternative splicing regulatory proteins within cells comprising introducing into cells polynucleotide sequences that bind to the RNA alternative splicing regulatory proteins, modifying within cells the activity of the RNA alternative splicing regulatory proteins by binding the polynucleotides to the RNA alternative splicing regulatory proteins, causing an effect within cells in the processing of RNA by modifying the activity of the RNA alternative splicing regulatory proteins, and determining the effect resulting from themodification of the activity of the RNA alternative splicing regulatory proteins. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide by electroporation. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences

by applying the polynucleotide sequences to the surface of the cells packaged in liposomes. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells along with a detergent.

The step of modifying within cells the activity of RNA alternative splicing regulatory proteins may further comprise regulating the activity of RNA alternative splicing regulatory proteins. The step of modifying within cells the activity of RNA alternative splicing regulatory proteins may further comprise reducing the activity of RNA alternative splicing regulatory proteins. The step of modifying within cells the activity of the RNA alternative splicing regulatory proteins may further comprise blocking the activity of the RNA alternative splicing regulatory proteins. The step of modifying within cells the activity of the RNA alternative splicing regulatory proteins may further comprise binding the polynucleotides either reversibly, or irreversibly.

As embodied herein, the step of determining the effect in the processing of RNA may further comprise determining the effect by phenotypic characteristics of the cells. The step of determining the effect in the processing of RNA may further comprise determining the effect by Northern blot analysis of cell extracts.

As embodied herein, the cells may further comprise tissue culture cells, and non-human tissue culture cells. The cells may also further comprise non-human cells, non-human mammalian cells, and avian cells.

As embodied herein, the polynucleotide sequences may further comprise isolated

and purified RNA molecules, synthetic RNA molecules, and synthetic RNA analogs. The polynucleotide sequences may be single stranded.

An alternative preferred embodiment of the present invention is a method of modifying the activity of hnRNP A1 proteins within cells comprising introducing into cells polynucleotide sequences that bind to hnRNP A1 proteins, modifying within cells the activity of hnRNP A1 proteins by binding the polynucleotides to the hnRNP A1 proteins, causing an effect within cells in the processing of RNA by modifying the activity of the hnRNP A1 proteins, and determining the effect resulting from the modification of the activity of the hnRNP A1 proteins. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide by electroporation. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequences to the surface of the cells packaged in liposomes. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells along with a detergent.

The step of modifying within cells the activity of the hnRNP A1 proteins may further comprise regulating the activity of the hnRNP A1 proteins. The step of modifying within cells the activity of the hnRNP A1 proteins may further comprise reducing the activity of the hnRNP A1 proteins. The step of modifying within cells the activity of the hnRNP A1

proteins may further comprise blocking the activity of the hnRNP A1 proteins. The step of modifying within cells the activity of the hnRNP A1 proteins may further comprise binding the polynucleotides either reversibly, or irreversibly.

As embodied herein, the step of determining the effect in the processing of RNA may further comprise determining the effect by phenotypic characteristics of the cells. The step of determining the effect in the processing of RNA may further comprise determining the effect by Northern blot analysis of cell extracts.

As embodied herein, the cells may further comprise tissue culture cells, and non-human tissue culture cells. The cells may also further comprise non-human cells, non-human mammalian cells, and avian cells.

As embodied herein, the polynucleotide sequences may further comprise isolated and purified RNA molecules, synthetic RNA molecules, and synthetic RNA analogs. The polynucleotide sequences may be single stranded.

An alternative preferred embodiment of the present invention is a method of influencing splice choice in RNA within cells comprising introducing into cells polynucleotide sequences that bind to RNA splicing regulatory proteins, modifying within cells the activity of the RNA splicing regulatory proteins, modifying within cells the activity of the RNA splicing regulatory proteins by binding the polynucleotides to the RNA splicing regulatory proteins, causing an effect within cells in the processing of RNA by modifying the activity of the RNA splicing regulatory proteins, and determining the effect in the processing of RNA resulting from the modification of the activity of the RNA splicing

regulatory proteins. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide by electroporation. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequences to the surface of the cells packaged in liposomes. The step of introducing into cells polynucleotide sequences may further comprise introducing the polynucleotide sequences by applying the polynucleotide sequence to the surface of the cells along with a detergent.

The step of modifying within cells the activity of the RNA splicing regulatory proteins may further comprise regulating the activity of the RNA splicing regulatory proteins. The step of modifying within cells the activity of the RNA splicing regulatory proteins may further comprise reducing the activity of the RNA splicing regulatory proteins. The step of modifying within cells the activity of the RNA splicing regulatory proteins may further comprise blocking the activity of the RNA splicing regulatory proteins. The step of modifying within cells the activity of the RNA splicing regulatory proteins may further comprise binding the polynucleotides either reversibly, or irreversibly.

As embodied herein, the step of determining the effect in the processing of RNA may further comprise determining the effect by phenotypic characteristics of the cells. The step of determining the effect in the processing of RNA may further comprise determining the effect by Northern blot analysis of cell extracts.

As embodied herein, the cells may further comprise tissue culture cells, and non-human tissue culture cells. The cells may also further comprise non-human cells, non-human mammalian cells, and avian cells.

As embodied herein, the polynucleotide sequences may further comprise isolated and purified RNA molecules, synthetic RNA molecules, and synthetic RNA analogs. The polynucleotide sequences may be single stranded.

Example 1

Inhibition of hnRNP A1 function

To test the hypothesis that hnRNP A1 is involved selecting the correct splice choice variant of FGFR2, exon competition assays were designed. The following competition studies were performed with the hypothesis that the ASF/SF2 and hnRNP A1 are antagonists and make splice choice selections in the third exon loop of the *fgfr2* pre-mRNA (Caceres *et al.*, 1998; Caceres *et al.*, 1994; Mayeda and Krainer, 1992; Yang *et al.*, 1994). ASF/SF2 preferentially selects the proximal splice choice *IIIb* exon, while hnRNP A1 selects the distal splice variant *IIIc* by binding to the ESS sequence in the *IIIb* exon (Del Gatto-Konczak *et al.*, 1999). Splice choice selection is based on the relative concentration of the two splicing factors so that high ASF/SF2 concentration yields the *IIIb* mRNA splice variant and production of the FGFR2IIIb isoform of the receptor and should result in a fused cartilage phenotype rather than discrete nodules.

The distal tips of stage 25 chicken leg buds were electroporated with the 138 base sense transcript from the chicken *fgfr2IIIb* mRNA containing the ESS corresponding to

exon 8. As a control, leg buds were also electroporated with the 141 base sense transcript from the chicken *fgfr2IIIc* mRNA corresponding to exon 9. Transfected cultures were grown in serum-free medium and stained as previously described. Cultures transfected with exon 9 displayed discrete nodules as expected (Downie and Newman, 1994). When cultures were transfected with exon 8 containing the ESS a continuous mass of cartilage formed with extensive cartilage formation in the central region again with few residual nodules at the periphery (Fig. 5a). When cultures were transfected with exon 8 transcript, a continuous mass of cartilage formed in the central region, again with few residual nodules at the periphery (Fig. 5b). This was most likely due to the interaction of the endogenous splice choice factor, in this case, hnRNP A1, with the exogenous exon 8 transcript containing the ESS sequence. Although hnRNP A1 is also involved in making splice choices of its own pre-mRNA, this process is accomplished by binding at intronic sites rather than exonic sites, as with FGFR2. The transfection of cultures with exogenous exon 8 would therefore not be expected to interfere with the correct processing of hnRNP A1 pre-mRNA.

By the hypothesis presented above, the lack of availability of hnRNP A1 should lead to expression of the incorrect *kgfr (IIIb)* splice variant rather than the correct *bek (IIIc)* FGFR2 isoform. As noted above, this missplicing also occurs in certain severe Apert syndrome cases as a result of *Alu*-element insertions in the FGFR2 gene (Oldridge *et al.*, 1999).

Example 2

The ribonucleoprotein hnRNP A1 plays a role in both RNA splice site selection and nucleus-to-cytoplasm transport of mRNA. In its capacity as a splicing factor, this protein modulates 5' splice site selection in a group of gene products containing a well-characterized RNA sequence determinant, the exonic splicing silencer (ESS). The HIV type 1 tat protein, the FGFR2 (Caputi *et al.*, 1999; Del Gatto-Konczak *et al.*, 1999; Mayeda *et al.*, 1994), and hnRNP A1 are among the pre-mRNAs that undergo differential splicing (Chabot *et al.*, 1997). In its role in nucleus-to-cytoplasm transport, hnRNP A1 acts as a "shuttle" protein, and is characterized by a novel amino acid motif found at its C-terminus termed M9, which contains both nuclear localization and nuclear export activities (Nakielny and Dreyfuss, 1997a; Nakielny *et al.*, 1999).

hnRNP A1 transcripts and protein are localized in whole and sectioned 4½–12 day embryos, as well in limb bud micromass cultures. In whole and sectioned tissue, expression has been detected in the skin, heart, gizzard, liver, lung, vertebral bodies, neural tissue, intestine, kidney tubules, and developing limb cartilage. In the developing cartilage of the vertebrae and limbs hnRNP A1 protein is initially present in precartilage cell condensations and persists in early chondrocytes. Earlier studies have looked at the distribution of hnRNP A1 in various differentiated cell types as well as in developing germ line cells of postnatal mice (Faura *et al.*, 1995; Kamma *et al.*, 1995). These studies demonstrated that hnRNP A1 was expressed at higher levels in earlier stages of spermatocyte development (Kamma *et al.*, 1995). The studies described in this thesis represent the first systematic analysis of hnRNP A1's expression during embryogenesis.

The functional significance of the stage-dependent hnRNP A1 expression patterns seen during development is dependent in part on the role of this protein in alternative splicing. One developmentally important gene whose transcript is alternatively spliced with the participation of hnRNP A1 is fibroblast growth factor receptor 2 (FGFR2) where it is involved in the choice of exon 9 rather than exon 8 (Del Gatto-Konczak *et al.*, 1999). Because (i) FGFR2 has a known pattern of spatiotemporal expression during appendicular chondrogenesis (Peters *et al.*, 1992; Lizarraga *et al.*, 1999; Szebenyi and Fallon, 1999), (ii) incorrect exon 8/exon 9 choice in FGFR2 in humans has known morphological consequences to the limb skeleton (Oldridge *et al.*, 1999), and (iii) the studies described herein show hnRNP A1 to have a distribution in the developing limb coincident with that previously found for FGFR2, the remainder of the work described was directed towards testing whether interference with hnRNP A1 synthesis or function had the effects predicted on the basis of our current understanding of FGFR2 function in limb skeletal patterning.

The fibroblast growth factors (FGFs), of which 19 structural vertebrate polypeptide homologs have been identified (Ornitz, 2000), are major modulators of embryonic development. Their roles include the formation of the primary body and neural axes, limbs and other structures including the heart, liver, muscle, head and face, teeth, lung, pancreas, skin, salivary glands, as well as the trophoectoderm and the inner cell mass of pregastrulating mammalian embryos (Arman *et al.*, 1999; Burke *et al.*, 1998; Chan and Thorogood, 1999; De Moerlooze *et al.*, 2000; Eckenstein, 1994; Hajihosseini and Dickson, 1999; Jang *et al.*, 1997; Jung *et al.*, 1999; Kettunen *et al.*, 1998; Olwin *et al.*, 1994;

Patstone *et al.*, 1993; Szebenyi and Fallon, 1999; Wilke *et al.*, 1997; Zhu *et al.*, 1999). By affecting gene expression, FGFs coordinate cellular functions including survival, replication, differentiation, adhesion and motility (Szebenyi and Fallon, 1999).

FGFs bind to cell surface receptors including (i) FGF receptor tyrosine kinases 1-4, (ii) a cytosine rich FGF receptor, and (iii) heparan sulfate proteoglycans (HSPGs) (Jang *et al.*, 1997; Lin *et al.*, 1999; Olwin *et al.*, 1994; Ornitz, 2000). Signaling through FGFs requires both the high affinity FGFRs and low affinity HSPGs to form an active complex (Aviezer *et al.*, 1999; Eckenstein, 1994; Lin *et al.*, 1999; Olwin *et al.*, 1994; Ornitz, 2000). In humans, mutations in FGFR1, -2, and –3 lead to five distinct craniosynostosis syndromes including Apert syndrome which arises from mutations in FGFR2 (Chan and Thorogood, 1999; Oldridge *et al.*, 1997; Oldridge *et al.*, 1995; Oldridge *et al.*, 1999). Additional abnormalities are seen in the limbs, skin, teeth, and CNS of these individuals (Chan and Thorogood, 1999).

Differential splicing of FGFR2 at the third Ig-like loop produces the FGFR2IIIb and IIIc splice variants which are active in epithelial-mesenchyme differentiation (Arman *et al.*, 1998; Orr-Urtreger *et al.*, 1993). Earlier studies have shown that FGFR2IIIb is preferentially expressed in epithelial tissues and that FGFR2IIIc is expressed in the mesenchyme (Orr-Urtreger *et al.*, 1993).

Proper patterning of the vertebrate limb relies on expression of hnRNP A1 and, as a consequence, the appropriate splice form of FGFR2. Previous work done by S. Downie (Ph.D. thesis, New York Medical College) showed that micromass leg cultures in the

absence of ectoderm and in the presence of serum produced a continuous sheet of cartilage. In contrast, cells cultured in the presence of ectoderm produced individual nodules with regions of perinodular inhibition. Leg micromass cultures that were devoid of ectoderm but with the exogenous addition of the growth factor FGF2 formed discrete nodules with very large regions of inhibition. These findings suggest that limb skeletal pattern depends, in part, on an activator-inhibitor interaction (Newman *et al.*, 1981a; Newman and Tomasek, 1996).

When limb bud precartilage mesenchymal cells are plated as high density micromass cultures, cells begin to condense and by day 6 after plating, will form cartilage nodules, which stain with alcian blue.

Based on previous data the following model is suggested for the phenomenon described in micromass cultures:

1. Limb bud mesenchymal cells anchor and produce the diffusible molecule TGFβ (Leonard *et al.*, 1991; Miura and Shiota, 2000).
2. TGFβ auto-stimulates its own production (Van Obberghen-Schilling *et al.*, 1988).
3. TGFβ also stimulates the production of hnRNP A1
4. hnRNP A1 determines the splice choice from the *IIIb*(*kgfr*) form to the *IIIc*(*bek*) form in FGFR2 pre-mRNA (Del Gatto-Konczak *et al.*, 1999).
5. FGFR2 *IIIc* isoform is stimulated by FGFs and mediates the production of an unknown "inhibitor" which then downregulates TGFβ expression, allowing for the formation of spaces between the cartilage nodules.

When limb bud micromass cultures were treated with exogenous TGFβ they formed precocious cartilage nodules by 72 h. Immunofluorescent detection of hnRNP A1 in these cultures shows that protein expression is within the condensing region. Confocal microscopy of normal 72 h cultures shows that all cells within the condensations are expressing high levels of the hnRNP A1 protein.

When leg limb bud tips were electroporated with hnRNP A1 antisense transcripts perinodular regions of inhibition were no longer detected and a continuous sheet of cartilage was seen. Western blot analysis of electroporated cultures confirmed that there was a decrease in the amount of hnRNP A1 protein detected in antisense treated cultures at 48 h. Furthermore, when antisense RNA directed against hnRNP A1 was introduced into a developing wing bud *in ovo*, a large mass of ectopic cartilage formed. These results indicate that lack of hnRNP A1 during the period of limb pattern formation lead to a lack of normal inhibition around developing cartilage elements, consistent with its role in the model described above.

When limb bud tips were electroporated with FGFR2 exon 8, which contains the ESS consensus sequence for hnRNP A1 binding, the cartilage pattern was similar to that of cultures that were treated with hnRNP A1 antisense RNA. The model outlined above would predict that excess ESS sequence would interfere with the normal binding of hnRNP A1 to its target sequence in FGFR2. Since hnRNP A1 and SF2/ASF are antagonists for exon splicing the loss hnRNP A1 function will lead to the incorrect splice choice and the FGFR2IIIb instead of FGFR2IIIc. As predicted, transfection of exon 8 (but not exon 9)

reduced the production of perinodular inhibitory activity (Fig. 5). Preliminary Northern blot results indicate that transfecting cultures with exon 8 interferes with the splicing of FGFR2 mRNA. *In vivo*, individuals with *Alu* elements in *exon IIIC* (exon 9) splice only *exon IIIb* (exon 8) and present with syndactyly (Oldridge *et al.*, 1999).

These results also bear on the possible role of hnRNP A1 at the other sites in which it has been found during embryogenesis. While this splicing factor undoubtedly has a variety of targets, other than its role in self-splicing its role in FGFR2 is best understood. Therefore the spatiotemporal coordination of the expression of FGF receptors and hnRNP A1 will be emphasized in the following discussion.

In embryonic mice, *FGFR2IIIb* and *IIIc* mRNA splice variants have been localized in various tissues including the developing lung bud were the *IIIb* isoform was localized in the bronchial epithelia and limb ectoderm. The *IIIc* isoform was localized in the mesenchyme of the lung and developing limb buds (Arman *et al.*, 1999). De Moerlooze *et al.* (2000) found that null mice for the FGFR2IIIb isoform were viable until birth but displayed severe limb, lung, and anterior pituitary gland defects with tissues undergoing apoptosis. Abnormalities were also detected in the salivary glands, inner ear, teeth, skin, and skull.

Chan and Thorogood (2000) looked at mutations in FGFR1 and 2 in 6-8 week human embryos and found that FGFR1 and FGFR2IIIb and IIIc isoforms were expressed in the enamel epithelium and papilla mesenchyme of the tooth germ. In addition, both genes are expressed in the cortical layer of the brain. Hajihosseini and Dickson (1999)

showed that embryonic day 15 cultured rat cortical cells initially express *FGFR1*, *-2*, and *–3IIIc* isoforms but within 16 hours post culturing they downregulated the *FGFR2IIIc* splice variant. Wilke *et al.* (1997) looked at the role of FGFs in skull, brain, and facial prominence in differential growth in chicken embryos and correlated these findings to FGFR mutations in humans.

Using *in situ* hybridization techniques, Kettunen *et al.* (1998) analyzed *FGFR1* and *2* expression patterns in mouse teeth. They found that the *FGFR1IIIc* splice variant was expressed in both the dental epithelium and mesenchyme while *FGFR2IIIc* was restricted to the dental follicle mesenchyme. They suggested that FGFs regulated differentiation and secretory functions in both odontoblasts and ameloblasts through the FGFR1IIIc signaling pathway, with additional signaling through the FGFR2IIIc isoform in the ameloblasts.

Walsh and Mason (2000) looked at the expression of *FGFR1*, *-2*, and *–3* transcripts in early neural development in chicken embryos. Other studies showed that FGFs can induce neural tissue to form from unstimulated epiblast (Alvarez *et al.*, 1998; Storey *et al.*, 1998; Walshe and Mason, 2000). Studies investigating the role of FGF4 and 8 demonstrated that FGF4 regulates the specification of the midbrain (Shamim *et al.*, 1999) while FGF8 regulates isthmus and midbrain proliferation and polarization (Crossley *et al.*, 1996; Martinez *et al.*, 1999; Sheikh and Mason, 1996). During neural induction all three *FGFR*s transcripts were localized with the *FGFR1IIIc* isoform predominantly detected in the neural plate and mesendodermal cells. Both *FGFR2IIIb* and *IIIc* were localized in the anterior primitive streak and in the neural plate region close to the head process while

FGFR3IIIc was localized in the lateral ectoderm anterior to Henson's node. *In situ* hybridization of Hamburger-Hamilton stage 17 embryos showed that FGFR2 was expressed in the mesonephric ducts as well as throughout the ectoderm. Finally, *FGFR2* transcripts were localized to the ectoderm and AER of the developing limb bud of stage 17 embryos.

Patstone *et al.* (1993) suggested that expression of FGFR1, -2, and –3 in various chicken embryonic tissues including the developing bones, skeletal-, cardiac-, and smooth muscle, as well as areas of the brain, may represent cell-type specific regulation and that the ligand-receptor interaction may likely be controlled by spatiotemporal constraints.

Zhu *et al.* (1999) examined the role of FGFs on proliferation and terminal differentiation of precardiac mesodermal cells and endodermal cells in the heart forming region of stage 6 chicken embryos. Jung *et al.* (1999) looked at the relationship of FGF1, -2, and –8 produced by cardiac mesoderm and the induction of gut-derived organs, including the liver in embryonic mice. Findings indicate that the FGF signaling pathway is necessary for the formation of the heart and that the FGF growth factors produced by the heart are also necessary for the induction of the foregut endoderm into the liver. Development of the exocrine pancreas requires FGFs and FGFR2IIIb (Miralles *et al.*, 1999). Pancreatic explants from embryonic day 11.5 rat embryos consisting of epithelium and mesenchyme showed a two fold decrease in size when treated with antisense FGFR2IIIb oligonucleotides (Miralles *et al.*, 1999).

Finally, Arman *et al.* (1998) mutated the FGFR2 gene and found that homozygous

mutant mouse embryos died hours after implantation. In culture, mutants formed a layer of trophoblast cells but did not maintain the inner cell mass nor produce visceral endoderm. These studies showed that FGFR2 was necessary for the outgrowth, differentiation and maintenance of the inner cell mass. Additional studies involving mammalian embryonic development showed that FGF4 was expressed in early cleavage (Rappolee *et al.*, 1994) (Arman *et al.*, 1999) and continued through the blastocyst, egg cylinder, and primitive streak stages (Arman *et al.*, 1999; Niswander and Martin, 1992). After implantation the main axes of the body form as well as the extraembryonic tissue precursors (Arman *et al.*, 1999; Gardner, 1983) and one of the earliest acting receptors in embryonic pattern formation may be FGFR2 (Arman *et al.*, 1999; Orr-Urtreger *et al.*, 1991).

Based on the localization of FGFRs in various embryonic tissues including the heart, liver, skin, developing nervous system, and extraembryonic membranes and the localization of hnRNP A1 protein in the same organs and tissues of the embryonic chicken, it may be hypothesized that hnRNP A1 plays a role in regulating the splice choice variants of FGFR2, and perhaps the other FGF receptors. If hnRNP A1 plays this role it would constitute a major coordinator of post-transcriptional cell type diversification during development. Certainly other targets of hnRNP A1 activity will come to light in the next few years, as well as information on the developmental roles of other nonconstitutive splice choice factors such as hnRNP A2/B1 and D classes.

Example 3

Previous work has shown that exon 8 of FGFR2 contains a splicing silencer that

interacts with the splicing factor hnRNP A1 (Del Gatto-Konczak F, Olive M, Gesnel MC, Breathnach R, 1999). The introduction of mimics (competitive antagonists) of endogenous splicing silencers can alter splicing pathways and bring about cell and tissue phenotypes characteristic of the altered pathway.

Leg bud mesenchyme cells grown in culture normally form an array of separate cartilage nodules, where each nodule is the approximate size of the cartilage primordia that lead to isolated skeletal elements during development (Downie SA, Newman SA. Morphogenetic differences between fore and hind limb precartilage mesenchyme: relation to mechanisms of skeletal pattern formation. Dev Biol 1994; 162:195-208; Downie SA, Newman SA. Different roles for fibronectin in the generation of fore and hind limb precartilage condensations. Dev Biol 1995; 172:519-30).

Fig. 3 illustrates how Apert syndrome is caused by mutations in FGF receptor 2 (FGFR2) and leads to severe syndactyly of hands (*a*) and feet (*b*). This is seen in an X-ray as a bony bridge between the fingers where there is usually open space (*c*). Panels (*a*) and (*b*) are from Park WJ, Theda C, Maestri NE, et al. Analysis of phenotypic features and FGFR2 mutations in Apert syndrome. Am J Hum Genet 1995; 57:321-8. (1995); panel (*c*) is from Wilkie AO, Slaney SF, Oldridge M, et al. Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. Nat Genet 1995; 9:165-72. (1995).

Fig. 4 illustrates that some of the most severe forms of syndactyly are seen in mutations

in which FGFR2 is misspliced (patients 1 and 2) leading to the inclusion of exon 8, which specifies protein domain Ig IIIb, in the final mRNA instead of exon 9, which specifies protein domain Ig IIIc (diagram from Oldridge M, Zackai EH, McDonald-McGinn DM, et al. De novo alu-element insertions in FGFR2 identify a distinct pathological basis for Apert syndrome. Am J Hum Genet 1999; 64:446-61.). The form containing Ig IIIc is the normal one for the mesenchymal cells that form the limb skeleton during development (Orr-Urtreger A, Bedford MT, Burakova T, et al. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev Biol 1993; 158:475-86).

Fig. 5a illustrates that chicken leg bud mesenchymal cells were transfected with FGFR2 exon 9 sense strand RNA (left) and exon 8 sense strand RNA (right) and grown *in vitro* for 6 days until cartilage was evident (blue stain). (Exons 8 and 9 are 138 amd 141 bases in length, respectively, and contain no 5' translation initiation sites). As predicted by the invention disclosure, exon 8, which contains the hnRNP A1 binding site, led to a phenotype analogous to that seen in Apert syndrome, in which the skeletal elements are fused and joined, rather than separate. Transfection with the control RNA, exon 9, which does not bind to hnRNP A1, left the nodules isolated from one another, as in untransfected cultures.

Fig. 5b illustrates that a developing chicken wing was transfected with FGFR2 exon 8. In this case there was a thickening of the humerus (arrow) and appearance of developing skeletal tissue between the radius and ulna (arrowhead), again similar to Apert syndrome in which ectopic bone forms as a result of the missplicing of FGFR2.

Fig. 5c illustrates that a Northern blot was performed using poly A RNA from leg bud mesenchyme that had been transfected with FGFR2 exon 8 or exon 9 sense strand RNA. Non-transfected cells (N) produced an RNA corresponding to the molecular size of FGFR2 mRNA (arrowhead) that was detected by a radioactive exon 9-specific probe (lane 1), but was not detected by an exon 8-specific probe (lane 2). In contrast, cells transfected with exon 8 RNA (E8) produced an RNA of the correct molecular weight that was detected by the exon 8-specific probe, marking it as abnormally spliced (as suggested by the Apert-like phenotypes of the exon 8-transfected cells and limb in Fig. 5a and 5b). Cells transfected with exon 9 (E9) produced no abnormally-spliced FGFR2 RNA containing exon 8.

It will be apparent to those skilled in the art that various modifications and variations can be made in the construction, configuration, and/or operation of the present invention without departing from the scope or spirit of the invention. For example, in the embodiments mentioned above, various changes may be made to the polynucleotide sequence and methods without departing from the scope and spirit of the invention. Further, it may be appropriate to make additional modifications or changes to the length and/or structure of the polynucleotide sequences without departing from the scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of the invention provided they come within the scope of the appended claims and their equivalents.